Evaluation of antiviral activity of essential oil of *Trachyspermum Ammi* against Japanese encephalitis virus

Soumen Roy, Pratibha Chaurvedi, Abhay Chowdhary

Department of Virology, Haffkine Institute for Training, Research and Testing, Mumbai, Maharashtra, India

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ABSTRACT

Background: Japanese encephalitis is a leading form of viral encephalitis, prevalent mostly in South Eastern Asia caused by Japanese encephalitis virus (JEV). It is transmitted by the mosquitoes of the *Culex* sp. The disease affects children and results in 50% result in permanent neuropsychiatric disorder. There arises a need to develop a safe, affordable, and potent anti-viral agent against JEV. This study aimed to assess the antiviral activity of ajwain (*Trachyspermum ammi*: Umbellifereae) essential oil against JEV. **Materials and Methods:** Ajwain oil was extracted by distillation method and *in vitro* cytotoxicity assay was performed in vero cell line by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay method. JEV titer was determined by plaque assay and *in vitro* antiviral activity of ajwain oil was quantified by the plaque reduction neutralization test (PRNT). **Results:** Cytotoxic concentration of the oil was found to be 1 mg/ml by MTT assay. The titer of the virus pool was found to be 50×10^7 PFU/ml. we observed 80% and 40% virus inhibition in 0.5mg/ml of ajwain oil by PRNT method in preexposure treatment and postexposure treatment (antiviral activity), respectively. **Conclusion:** Our data indicate ajwain oil has potential *in vitro* antiviral activity against JEV. Further, the active biomolecule will be purified and evaluated for anti-JEV activity and also to scale up for *in vivo* trial to evaluate the efficacy of ajwain oil in future.

Key words: Antiviral, ajwain oil, cytotoxicity, Japanese encephalitis virus

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INTRODUCTION

Japanese encephalitis (JE) is caused by Japanese encephalitis virus (JEV) which belongs to the flaviviridae family and is the most important encephalitis causing virus in Asia. JEV is reported to cause 35–50 thousand cases and 10–15 thousand deaths annually.[1] JEV is small RNA virus transmitted by mosquitoes and ticks. JEV generally affects small children (<15 years) and elderly people (>65 years), who have weak immune system and hence are vulnerable.[2] JEV leads to major outbreaks in tropical regions of Asia with China, Japan, Korea, Philippines, Southeastern Asia and India.[3] There is no reported marketed antiviral drug against JEV although many compounds like a combination of N-methylisatin-β-thiosemicarbazone derivative (SCH16) with ribavirin griffithsin (GRFT) demonstrate the *in vitro* antiviral activity against JE.[4-6] Extensive animal trails are needed to develop an antiviral drug against JEV. Medicinal plants have been widely used to treat a variety of infectious and noninfectious diseases and represent an abundant source of new bioactive secondary metabolites. *Trachyspermum ammi* is a plant of Umbelliferae family and is much used as a medical plant in Ayurvedic medicine, and its essential oil are reported to possess hypolipidemic, antihypertensive, antispasmodic, antilithiasis and diuretic properties. It also possesses antimicrobial, nematicidal, antihelminthic, antifilarial activities.[7] The essential oil (2.5% to 5% in the dried fruits) is dominated by thymol (35% to 60%) furthermore, α-pinene, p-cymene, limonene, which majorly contributes to its curative properties.[8] *T. ammi* commonly known as ajwain was selected to evaluate its anti-JE activity *in vitro*. Ajwain essential oil has been reported to be effective as antifungal, antibacterial, and antiviral agent.[9] This made a basis to screen the essential oil of *T ammi* for *in vitro* anti JE activity. The study is not well-documented until date, hence described study was conducted.

Address for correspondence:
Soumen Roy, Department of virology, Haffkine Institute for Training, Research and Testing, Mumbai, Maharashtra, India.
E-mail: soumenroy118@gmail.com

MATERIALS AND METHODS

The seeds *T. ammi* were collected from kelkar farm house, Mulund (east) and the seeds were air-dried and grinded into fine powder. Briefly, hydrodistillation method was carried out to extract the essential oil by adding 50 g of powdered material (seed) in 100 ml of water in a clavenger's apparatus for 10 h to obtain light yellow color essential oil (1% yield obtained).[9] Pre- and post-exposure treatment were performed of the essential oil against JEV strain and the plaque reduction, and antiviral activity was quantified by plaque reduction neutralization test (PRNT) method.[10]

Cell line and virus

African green monkey kidney (vero) cell lines were procured from National centre for cell science (Pune) and were grown in minimal essential medium (MEM) with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and gentamicin (10 μg/ml), and supplemented with 10% fetal bovine serum. The standard strain of JEV (JE 733913) was procured from National Institute of Virology, Pune. The virus stocks were stored at −80°C temperature for further use.

Virus titration

Japanese virus (JE) titration was performed by plaque assay. Vero cells were trypsinized, and a cell suspension containing 0.2× 10^6 cells/ml prepared in MEM containing 10% fetal calf serum (FCS) was dispensed in each well of the 24-well plate (Corning). The plate was incubated for 24 h at 37°C with 5% CO_2 to obtain a confluent monolayer. The spent medium from the cell monolayer was removed, and serial 10-fold dilution of the virus (0.1 ml) was added onto the cell monolayer and kept for absorption for 1 h at 37°C with intermittent shaking. The cell monolayer was covered using 1 ml of carboxymethyl cellulose (CMC) overlay medium (1 part of 1.8% CMC +1 part of 2× MEM with 2% FCS). The plate was incubated at 37°C in 5% CO_2 environment for 48 h. The assay was terminated by decanting the CMC overlay medium followed by a saline wash. The plates were stained with 1% amido black and incubated at room temperature (RT) for 45 min. The stain was washed under tap water and plaques were counted to obtain the virus titer.[10]

Cytotoxicity assay

The cytotoxic concentration (CC50) of ajwain oil (1%) was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Vero cells were trypnised and cultured onto 96 well plate at the density of 0.2 × 10^6 cells/ml. Once the monolayer is formed after 24 h, different concentrations (4 mg/ml to 0.1 mg/ml) of ajwain oil were serially diluted and added to each culture wells in triplicate and incubated at 37°C with 5% CO2 for 16 h. After incubation, the medium with ajwain oil was removed and 10% of 5 mg/ml MTT (100 μl) was added to each well onto the vero cells in 96 well plate and incubated for 4 h at 37°C. After incubation, MTT (100 μl) was removed, and the crystal formed (formazan) was solubilized by adding dimethyl sulfoxide to each well. The absorbance was read at 550 nm by an enzyme-linked immunosorbent assay reader.[11]

Antiviral assay

The ajwain oil was examined for the extent of the reduction of the plaque as compared to the virus control by PRNT method.

Pre-exposure treatment

Japanese encephalitis virus of 50 × 10^7 PFU/ml (50 plaque) was exposed to effective minimal CC50 (1 mg/ml) of ajwain oil for 1 h at 37°C. Then 100 μl of the mixture was added to the 24 well plate containing confluent monolayer of vero cells (0.2 × 10^6 cells/ml) and incubated at 37°C for 1 h. The cell monolayer was covered using 1 ml of CMC overlay medium (1 part of 1.8% CMC +1 part of 2 × MEM with 2% FCS). The plate was incubated at 37°C in 5% CO_2 environment for 48 h.

Post-exposure treatment

Japanese encephalitis virus of 50 × 10^7 PFU/ml (50 plaque) was added to confluent vero cell monolayer in 24 well plate and incubated for 1 h at 37°C. After incubation, the ajwain oil of minimal CC50 (1 mg/ml) was mixed in CMC overlay medium and added onto the virus infected vero cell monolayer. The plate was incubated at 37°C in 5% CO_2 environment for 48 h. The assay was terminated by decanting the CMC overlay medium followed by a saline wash. The plates were stained with 1% amido black and incubated at RT for 45 min. The stain was washed under tap water, and plaques were counted to obtain the virus titer.[10]

Statistical analysis

The assay proceeded on three independent replication (n = 3) for each test. *In vitro* cytotoxicity range (CC50) was calculated by logamathic method and percent plaque inhibition was calculated by two-tailed t-test with $P < 0.05$ as significance using graph pad prism v5.0 software.

RESULTS

Virus titration

Japanese encephalitis stock virus was serially diluted (10^{-1} to 10^{-9}) and was infected onto the confluent monolayer of vero cells (0.2 × 10^6 cells/ml) in 24 well plate. The titration of JEV was performed by plaque assay. The titer of the JEV stock was be 50 × 10^7 PFU/ml [Figure 1]. The virus

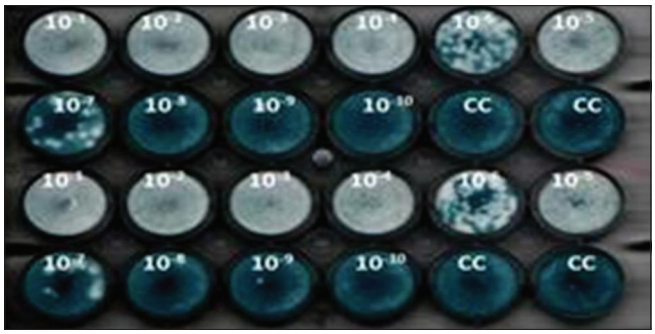

Figure 1: Plaque assay for Japanese encephalitis virus (JEV) vero cell line the titration of JEV was performed in vero cell line by plaque assay. The titre of the JEV stock was be 50 × 107 PFU/ml

titer was expressed in terms of plaque forming units/ml (PFU/ml) using the formula,

$$\text{PFU} / \text{ml} = \frac{\text{P} \times \text{D}}{\text{V}}$$

Where P is the number of plaques in a well, D is the reciprocal of the virus dilution and V is the volume of the virus dilution inoculated. The titrated virus stock was used for all the further experiments.

Cytotoxicity

The major prerequisite of the antiviral agent is safety. Thus, we tested the toxicity of ajwain essential oil in vero cells by MTT assay. The Figure 2 shows the log concentration of the ajwain oil in the range of 4 mg/ml to 0.1 mg/ml [Figure 2]. The 50% CC50 of ajwain oil was found to be 1 mg/ml. The CC50 value was then calculated using graph pad prism v5.0 software. Thus, the effective minimal concentration of ajwain oil was selected below 1 mg/ml for antiviral assay.

Antiviral assay

The *in vitro* anti-JE activity of ajwain oil was studied using PRNT method. The virus titre (50 × 10^7 PFU/ml) was used for the assay. In the pre-exposure treatment where the JEV (50 × 10^7 PFU/ml) was exposed for 1 h with ajwain oil of different concentration (1 mg/ml) below CC50 range. We observed that JEV was completely inhibited as no plaque was observed up to 1 mg/ml of whereas in 0.5 mg/ml 10 plaques was observed, and 40 plaques was reduced as compared to JEV control of 50 plaques. Thus, 80% virus inhibition was observed in 0.5 mg/ml in pre-exposure treatment. Whereas at 0.25 mg/ml and 0.125 mg/ml we observed no plaque reduction [Figures 3 and 4]. In post-exposure treatment, the JEV (50 × 10^7 PFU/ml) was allowed to adsorb onto the vero cells for 1 h and followed by MEM containing ajwain oil of varing concentration below CC50 range was added along with CMC (1:1). We observed JE was completely inhibited as no plaque was observed up to 1 mg/ml, whereas in 0.5 mg/ml 30 plaques

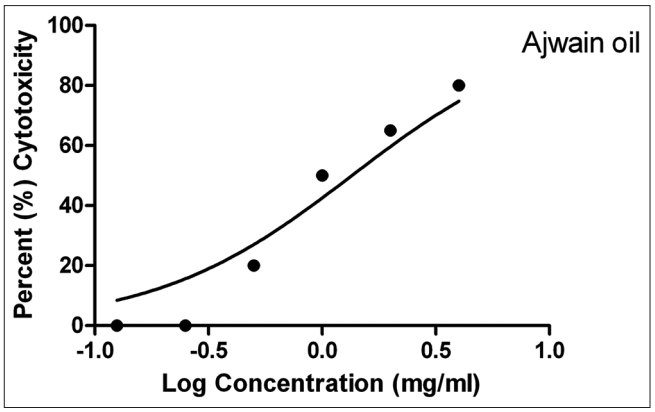

Figure 2: *In vitro* cytotoxicity of ajwain essential oil ajwain oil of different concentration (4–0.1mg/ml) was added in vero cell line for 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide based cytotoxicity assay. Cytotoxic concentration value was found to by 1 mg/ml and percent cytotoxicity is represented by logamathic method by graph pad prism v5.0 software

was observed, and 20 plaques was reduced as compared to JEV control of 50 plaques. Thus, 40% virus inhibition was observed in 0.5 mg/ml in postexposure treatment. Whereas, no plaques were reduced below 0.25 mg/ml and 0.125 mg/ml of ajwain oil [Figures 3 and 4].

DISCUSSION

Antiviral chemotherapy is an important component in the management of viral diseases. However at present potent antivirals for clinical usage are available only against limited number of the family of viruses (orthomyxoviridiae, herpesviridiae, retroviridiae). Traditional medicine continues to provide health coverage for over 80% of the world population, especially in the developing world. The past and the present are all full of living examples of discoveries of drugs, ranging from anti-cancer, anti-asthma, anti-diabetic, anti-hypertensive's and many others, which owe their origin to traditional medicine.[12] JEV remains a serious problem in many countries, coupled with long history of outbreak and epidemic. There a still no available antiviral drug against JEV.

In this study, we evaluated the antiviral activity of ajwain essential oil against JEV. *In vitro* cytotoxic activity of ajwain oil by MTT assay in vero cells is not well documented. The CC50 value of ajwain oil was found to be 1 mg/ml. We selected 50 plaques of JEV (50 × 10^7 PFU/ml) and the effective minimal concentrations of ajwain oil based on the CC50 range was used for the antiviral assay. In the minimal CC50 (1 mg/ml) of ajwain oil, we observed no plaque formation and complete virus inhibition as compared to virus control of 50 plaques in both pre-exposure and post-exposure treatment. In pre-exposure treatment, we observed a reduction of 40 plaques as compared to the

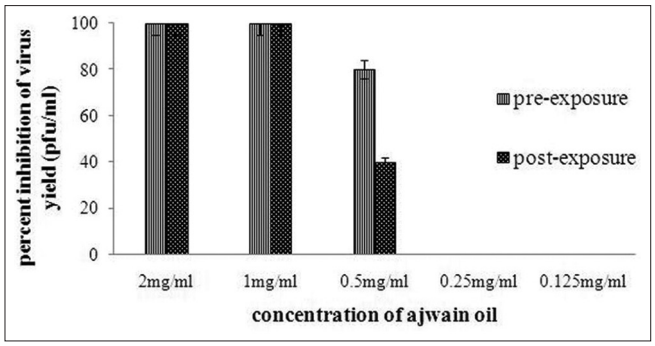

Figure 3: Percent inhibition of Japanese encephalitis virus at different concentration of ajwain oil *in vitro* antiviral activity of ajwain oil by pre-exposure treatment and post-exposure treatment was observed at different concentrations (2–0.1 mg/ml) in vero cell line. The viral inhibition was determined by using plaque reduction neutralization test method

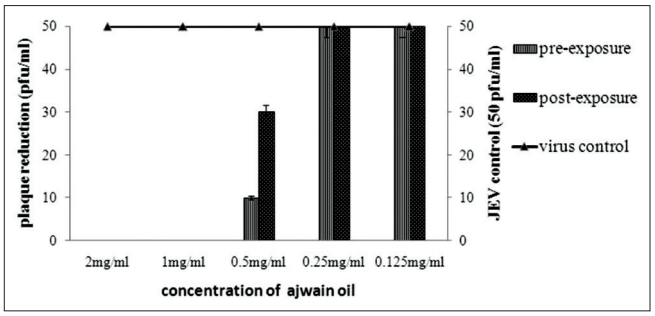

Figure 4: *In vitro* anti- Japanese encephalitis activity of ajwain oil pre-exposure treatment and post-exposure treatment of ajwain oil at different concentrations (2–0.1 mg/ml) as compared to Japanese encephalitis virus control (50 × 107 PFU/ml) of 50 plaque was inoculated in vero cells. The plaque reduction of pre- and post-exposure treatment of ajwain oil was observed by plaque reduction neutralization test method

JEV control (50×10^7 PFU/ml) of 50 plaques in 0.5 mg/ml of ajwain oil. Thus, 80% virus inhibition was observed in 0.5 mg/ml of ajwain oil, whereas no inhibition and observed below 0.5 mg/ml. In post-exposure treatment, we observed 20 plaque reduction as compared to the JEV control (50×10^7 PFU/ml) of 50 plaques in 0.5 mg/ml of ajwain oil. Thus, 40% virus inhibition was observed in 0.5 mg/ml of ajwain oil and no virus inhibition below 0.5 mg/ml of ajwain oil in post-exposure treatment.

A similar study had been conducted on *Isatis indigotica, a* herb which grows in China that is traditionally used for the clinical treatment of viral diseases like encephalitis, hepatitis, and influenza. Pretreatment of *I. indigotica* extracts significantly inhibit JEV replication *in vitro*. They obstruct JEV attachment and hence have potent antiviral activity. Combination of SCH16 with ribavirin GRFT demonstrates the *in vitro* antiviral activity against JE.[4-6]

Ajwain has been commonly used in traditional medicine systems for a variety of medicinal and pharmacological aspects. High amount of polyphenols are found in ajwain oil and it is rich in antioxidant activity. It is also used in food preservation.[13] Ajwain essential oil has a wide range of antibacterial and antifungal activity. *T. ammi* is a plant of Umbelliferae family and is much used as a medical plant in Ayurvedic medicine against diseases of the digestive tract and fever. The essential oil (2.5% to 5% in the dried fruits) is dominated by thymol (35% to 60%) furthermore, α-pinene, p-cymene, limonene.[14] Umbelliferae is well known for its essential oil content, which has been evaluated for its anti-viral activities in many studies.[15]

As ajwain oil which contains large amounts of thymol in its total essential oil have reported to exhibit bactericidal or bacteriostatic agents depending on the concentration.[16] Ajwain oil extracted from seed inhibits *Aspergillus niger* and *Curvularia ovoidea* at 5000 ppm as minimum inhibitory concentration.[17] Ajwian oil has a tremendous application as potential moiety as it has a variety of medicinal and pharmacological aspects and also used in various traditional and Persian medicine. It had been reported to have antiviral activity against hepatitis C virus (HCV). In a study, ajwain herb showed significant inhibitory effects on HCV protease.[18] Other antiviral activity of ajwain oil is not well explored. In our study, we observed *in vitro* antiviral activity of ajwain oil up to 0.5 mg/ml of the concentration in both pre-exposure and post-exposure treatment. There are no antiviral drugs against JEV till now. Minocyclin is an antibiotic but have showed good *in vitro* activity against JEV, but still up to research level, not available in market.[19,20] Our laboratory is actively involved in research of novel experimental moieties and exploring the antiviral activity of herbal and ayurvedic formulations against JEV and other viral encephalitis for the potential antiviral drug. Thus in our study, pre-exposure treatment of ajwain oil was found to be more effective in virus inhibition as compared to post-exposure treatment. The active molecule (thymol) present in ajwain oil need to be further screened for explore its potential for the antiviral drug in future.

CONCLUSION

The result of this study has explored the potential *in vitro* anti-JE activity of *T. ammi* (ajwain) oil by means of PRNT. Further, the active biomolecule will be purified and evaluated for its anti-JE activity and also to scale up for *in vivo* trial to evaluate the efficacy of ajwain oil for future use.

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